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DEMONSTRATIONS

A simple and inexpensive servo system for the control of length or tension of small muscles or stretch receptors

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We have developed a control system for 'clamping' and varying in a predetermined way either length or tension of the thoracic-coxal muscle receptor organ in the crab (see following Demonstration). The instrument could with minimal modification be employed in a variety of different contexts.

For the motive transducer (Fig. 1*a*) a small, inexpensive loudspeaker of low inertial mass and capable of fast rates of movement is used. The cone was removed and a stainless-steel tubular shaft fixed to the coil former. To monitor position of the shaft a transducer was fabricated using a metal foil strain gauge bonded to each face of two phosphor-bronze cantilever beams. The two beams provide a suspension point for the puller shaft, and also serve to increase sensitivity and minimize drift, since the four strain gauges are connected in bridge configuration. Dampers were fitted to reduce natural resonance in the beams.

The control system (Fig. 1*c*) is a four term closed loop type. The signal from the position transducer is amplified and subtracted from the demand input to give an error signal. This is then differentiated twice, providing signals proportional to the displacement, velocity and acceleration errors. These three signals are summed by a virtual earth amplifier with capacitive negative feed-back. This feed-back prevents loop gain exceeding unity for rates at which phase shift in the return section of the loop could cause positive feed-back to occur, and integrates any standing errors due to mechanical hysteresis in the motive transducer. The output from this control amplifier is applied to the motive transducer via a power amplifier.

In the case of tension clamping, the pull is applied to the muscle via a tension transducer (Fig. 1*b*). An Akers AE803 piezo-resistive strain gauge

assembly is used, this being essentially a silicon cantilever beam with strain gauges diffused into each face. The body of the transducer is clamped on the end of the puller shaft, a stainless-steel hook having been bonded on to the silicon beam for connexion to the receptor muscle. The two gauges are connected as a potentiometer and the output amplified. At present the

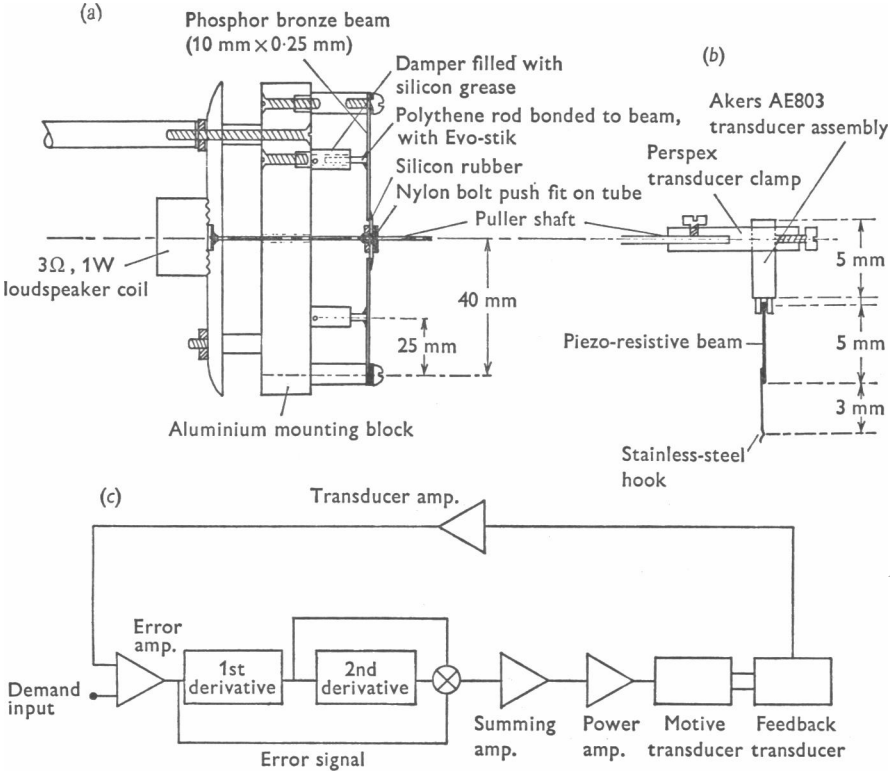

Fig. 1. Assembly sketches of (*a*) puller unit and (*b*) tension transducer. (*c*) Block diagram of puller servo system.

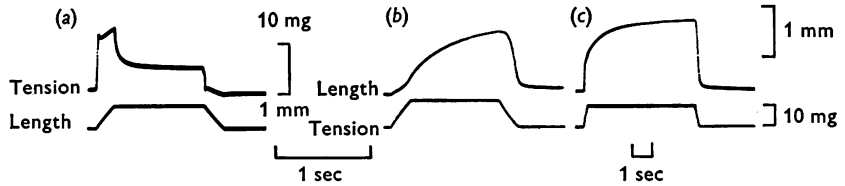

Fig. 2. (*a*) Tension change in the crab thoracic-coxal muscle receptor in response to a ramp-function length change. (*b*, *c*). Length changes resulting under 'tension clamp' conditions, with similar ramp-function inputs to that in (*a*).

transducer amplifier output is fed into the same control system as for length clamping. However, derivative feed-back appears not to be needed, presumably because of the visco-elastic behaviour of the muscle. This results in relatively slow movements of the puller for the required demand signals and provides velocity damping for the puller mass.

Examples of the performance of the puller servo as applied to the crab thoracic-coxal muscle receptor are illustrated in Fig. 2. In the length mode (*a*) the characteristic wave form of the 'tension response' to a ramp function length change closely resembles those previously reported (Bush & Godden, 1974). With tension clamping (*b*, *c*), the slow length changes required to produce ramp-shaped tension changes reflect the long time constant of the visco-elastic behaviour of this tonic muscle.

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Voltage clamping of non-impulsive afferents of the crab thoracic-coxal muscle receptor

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In view of its lack of afferent impulses, the thoracic-coxal muscle receptor organ of the crab provides the opportunity for the study of the mechanisms of mechano-sensory transduction without the necessity of using spike-blocking drugs. In order to distinguish primary transduction events from secondary voltage dependent conductance changes a technique for voltage clamping the sensory endings has been developed.

The intracellular potential is measured with a conventional KCl-filled glass micro-electrode, the extracellular potential with a KCl-agar reference electrode. The intra- and extracellular electrodes are connected to the non-inverting and inverting inputs respectively of a high-impedance differential amplifier (1, 2 and 3 in Fig. 1). Capacity compensation is available for the intracellular electrode. The output from the differential amplifier is summed (4) with a derivative component of itself (5 and 6) and a demand input. The resultant signal is amplified (4) and applied to an intracellular current injecting electrode to form a negative feed-back loop. The summing amplifier is lagged to prevent the loop gain exceeding unity for rates at which phase shift in the feed-back section of the loop could cause positive feed-back to occur. Controls for the loop gain and amount of derivative damping are provided on the front panel. A non-inverting high-impedance

amplifier (7) is available for recording from the current injecting electrode during impalement. A function switch selects either recording or injecting mode. An inverting amplifier (8) provides a virtual earth for the preparation and an output voltage proportional to the current flowing to ground. This voltage is then inverted (9) to give a positive output for conventional positive current flow.

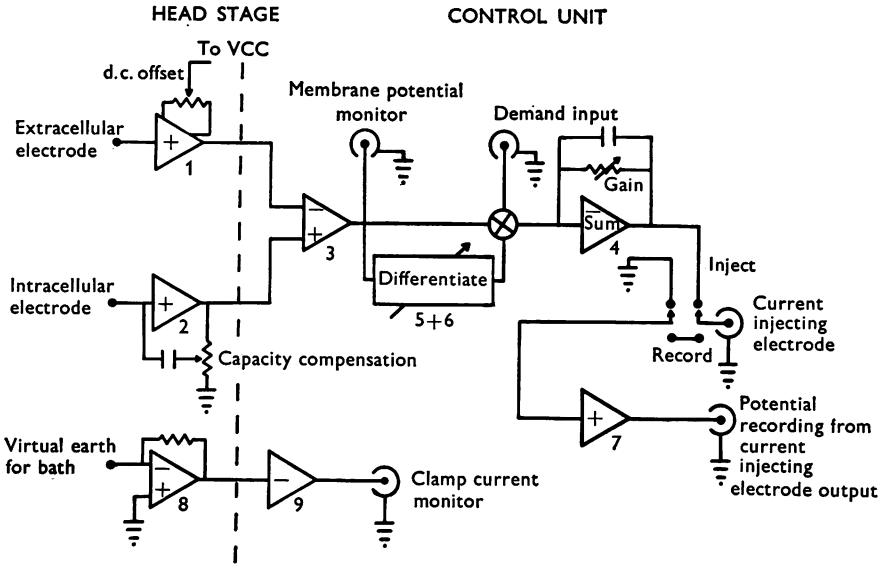

Fig. 1. Diagram of the voltage clamp system. 1, 2, 7 and 8 are Signetics NE536T FET-input amplifiers, and 3, 4, 5, 6 and 9 are 741-type amplifiers. Components cost less than £15 in total, excluding the +15, 0, −15 volt power supply.

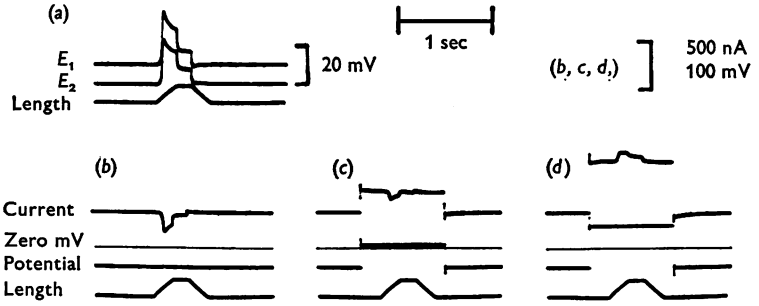

Fig. 2. (*a*) Receptor potentials in response to a ramp function length change recorded simultaneously with the voltage (E_1) and current (E_2) electrodes, prior to clamping the membrane at resting potential in (*b*). In (*c*) the membrane is clamped at a slightly positive potential, and in (*d*) at a positive level beyond the reversal potential for the receptor current.

The isolated muscle receptor system is pinned on to a Sylgard base in a temperature-controlled bath. Salines of different ionic composition, selected by a twelve-way tap (Partridge & Thomas, 1975), continuously perfuse the preparation. The current injecting and voltage recording microelectrodes are inserted into the in-series 'T' fibre whose short, thick dendrites innervate the basal end of the receptor muscle. Penetration is effected as close as possible to this dendritic region in order to minimize errors due to imperfect space clamping of the transducer region. Such errors are assumed to be small since the length constant of the axonal region of the T fibre may be up to 12 mm. Receptor potentials are generated in the T fibre by ramp stretches of the receptor muscle (Fig. 2*a*). The gain and damping of the feed-back system are then slowly increased, keeping membrane potential constant by introducing a d.c. demand signal, until no receptor potentials appear on the voltage recording channel. The clamp currents, which reflect inversely the receptor currents, are monitored on a second channel.

Receptor currents are inward going at normal resting potentials of about -60 mV, and have similar wave forms to the corresponding unclamped receptor potentials (Fig. 2*b*). The amplitude of the receptor current increases during hyperpolarizing clamp pulses, and decreases during depolarizing pulses (Fig. 2*c*). Reversal of the receptor current to an outward current occurs at an intracellular membrane potential between $+20$ and $+30$ mV (Fig. 2*d*). These observations suggest that the primary transduction process is a membrane conductance change, and that the receptor current is being carried predominantly by sodium ions, confirming previous observations (Roberts & Bush, 1971). Ion substitution experiments currently in progress aim to further elucidate the ionic basis of the sensory response.

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Transmitter enzymes and amino acid levels in sensory and motor nerves of the shore crab

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The nervous system of the crab offers a relatively simple system for neurophysiological and neurochemical studies. Of special interest in relation to current research on the crab muscle receptor (see Bush, Godden & Macdonald, 1975*a*, *b*) is the nature of the sensory transmitter released by the two large, decrementally conducting afferent neurones of this receptor. Despite their lack of regenerative impulses, depolarization of these afferent axons reflexly excites the 'extrafusal', promotor muscle (Bush & Cannone, 1973). It is thus also pertinent to identify the excitatory and possible inhibitory neuromuscular transmitters released by the promotor and antagonistic remotor motoneurones. Florey (1973) reviews the evidence that acetylcholine is the prevalent sensory transmitter in Crustacea, while glutamic acid and γ-aminobutyric acid (GABA) respectively may be the excitatory and inhibitory transmitters released by the motor nerves (cf. review by Kravitz, 1967).

Sensitive micro-enzyme assays developed by Emson & Fonnum (1974), allowing the simultaneous determination of two or three transmitter synthesizing enzymes, were described. Using these techniques, the presence of choline acetyltransferase, the enzyme responsible for the synthesis of acetylcholine, and glutamic acid decarboxylase (GAD) which synthesizes GABA, have been tested for in samples of sensory and motor nerves from the shore crab, *Carcinus maenas*. Lengths of nerve as long as possible were isolated from the small sensory nerves of the thoracic-coxal (T-C) muscle receptor (2–3 mm) and coxo-basal stretch receptor (5–8 mm), and from the promotor and remotor muscle nerves (3–5 mm). For comparison predominantly motor and sensory components were separated from the whole nerve trunk in several walking legs (20–50 mm).

The purely sensory T-C muscle receptor nerve contained one hundred times more choline acetyltransferase activity (579 ± 394 nmole/cm) than a similar sized motor nerve, the promotor muscle nerve ($5{\cdot}2 \pm 4{\cdot}7$ nmole/cm). Glutamic acid decarboxylase, on the other hand, was found in significant amounts in the promotor nerve ($6{\cdot}92 \pm 2{\cdot}35$ μmole/g, $n = 6$), and in smaller concentrations in the mixed remotor muscle nerve ($0{\cdot}314 \pm 0{\cdot}05$ μmole/g, $n = 3$) and mainly motor components of the leg nerve ($1{\cdot}03 \pm 0{\cdot}51$ μmole/g, $n = 15$). In contrast, no GAD activity was detectable in leg or coxal sensory nerves.

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We have also analysed samples of the same sensory and motor nerves for their amino acid content, using the micro-dansylation method of Joseph & Halliday (1975). This method depends upon the reaction of [^{3}H]dansyl chloride with the amino acids to form the derivative [^{3}H]dansyl amino acids. ^{14}C amino acids are used as internal standards to allow correction for incomplete and variable degrees of dansylation. Using this procedure, GABA was found in only trace amounts in the sensory nerves (coxal sensory nerves $0{\cdot}87 \pm 0{\cdot}33$ μmole/100 mg protein, $n = 3$; leg sensory nerve $0{\cdot}43 \pm 0{\cdot}31$ μmole/100 mg protein, $n = 6$), but in significantly higher amounts in the leg motor nerve ($4{\cdot}27 \pm 1{\cdot}68$ μmole/100 mg protein, $n = 8$). Levels of other amino acids, glutamic acid, glutamine, glycine, proline and taurine did not differ significantly.

The unequal distribution of the two enzymes and one amino acid observed would be consistent with acetylcholine being concentrated in the sensory nerves and GABA in the motor nerves studied, and hence with their probable role as synaptic transmitters in these nerves. While electrophysiological evidence for peripheral inhibitory motoneurones to the promotor and remotor muscles is as yet lacking, the present evidence for chemical as opposed to electrical synaptic transmission at the muscle receptor afferent terminals has important neurophysiological implications.

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Hydraulic model of nerve membrane recording

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Electrical models of the nerve membrane are of little help to many biology students who find the electrical events in nerve cells rather intimidating. A model was therefore made in which water pressure was analogous to membrane potential and taps controlled this pressure in the way that membrane permeability controls membrane potential. In this way the 'potentials' and the 'permeabilities' could be seen and their effects better understood. The model consists of three glass cylinders whose water level is set to form the equilibrium potentials of the three main

ions, E_{Na^+}, E_{K^+} and E_{Cl^-}. Each cylinder is connected by two rubber pipes to a manometer where the 'membrane potential' (E_M) is measured. On one rubber pipe is a screw clamp to set the resting 'permeability' while on the other is a spring clamp to make transient changes in the 'permeability' to a particular ion. To maintain the system in equilibrium a pump collects

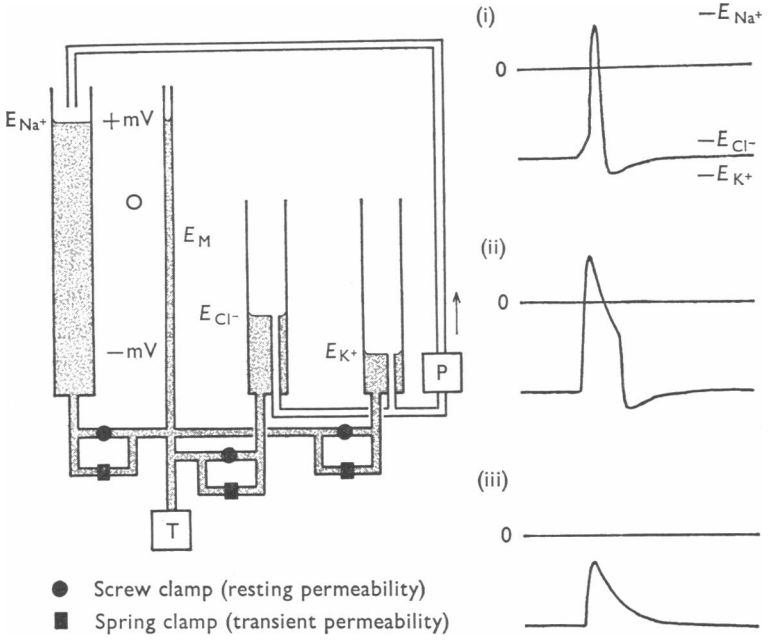

Fig. 1. Hydraulic nerve membrane model where the equilibrium potentials for Na^+, K^+ and Cl^- are represented by the water levels in three glass cylinders (E_{Na^+}, E_{K^+} and E_{Cl^-}) and the membrane potential (E_M) by that in a manometer. Membrane permeabilities are set by clamps on rubber tubes joining the glass cylinders for each ion to the manometer. A pump (P) keeps the system in equilibrium and a Sandborn model 267BC pressure transducer (T) in averaging mode provides an output for a pen recorder. The records on the right show (i) a nerve-type impulse, (ii) a cardiac-type impulse and (iii) an end-plate potential. These records were made at a chart speed of 2·5 cm per minute by manipulation of the transient Na^+ and K^+ clamps.

water from the overflows which set the levels of the K^+ and Cl^- cylinders and returns this to the Na^+ cylinder. Finally, to obtain a pen record of 'membrane potential', a transducer is connected to measure pressure in the manometer (see Fig. 1).

Examples of records from the model are shown in Fig. 1. A resting potential was first set up with K^+ 'permeability' and a slight Na^+ leakage 'permeability'. The impulse (i) was generated by sequential operation of

the Na^+ and K^+ transient clamps. The cardiac type impulse (ii) was obtained by delaying the K^+ clamp opening and the end-plate potential (iii) was obtained by opening the Na^+ and K^+ clamps together. The model offers wide possibilities for experiments on the effects of permeability changes on membrane potential.

A demonstration of some of the properties of obelin: a calcium-sensitive luminescent protein

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Recently Campbell (1974*a*, *b*) has reported that relatively large amounts of the calcium-sensitive photoprotein obelin (Morin & Hastings, 1971) can be obtained from the hydroid *Obelia geniculata* which is common off the South coast of Britain. The hydroid is associated with the brown seaweed *Laminaria* from which it can be readily removed. The photoprotein is extracted and purified by fairly straightforward protein isolation procedures (Campbell, 1974*b*). The protein, free of ammonium sulphate, and the majority of the EDTA is stored as a white lyophilized powder at $-70°$ C from which small batches of a concentrated solution of obelin can be prepared suitable for micro-injection. Generally, 0·1–0·3 μl. of a solution of obelin dissolved in 100 mM TES buffer, pH 7·3, was axially micro-injected into a cannulated barnacle muscle fibre and the light and tension responses were observed in response to electrical stimulation. The time course of these responses were similar to those observed in this preparation using the photoprotein aequorin internally (Ashley & Ridgway, 1970). The light emission responses were however much smaller in amplitude, even taking into account differences in the concentration of the two photoproteins. This finding could suggest that obelin has a lower apparent binding affinity for calcium than aequorin. The relative concentrations of the two photoproteins were assayed by comparing the total amount of light emitted from a given sample when maximally activated with calcium. These experiments also indicated that the maximum rate of utilization of obelin under these assay conditions was 3·9 s^{-1} at 23° C. In separate experiments, the barnacle myofibrillar bundle preparation (Ashley & Moisescu, 1973) was 'loaded' with obelin under paraffin oil (see legend to Fig. 1 for details and Ashley, Moisescu & Rose (1974)). The light and tension responses of the preparation were observed when it was activated with a calcium-EGTA buffer solution, pCa 5·9 (Fig. 1). The ability of obelin to respond at this relatively low pCa with a sub-maximally effective rate of utilization again suggests that obelin has a lower apparent affinity for calcium than

aequorin. A preliminary investigation of the light emission:pCa relationship for obelin indicates that the curve is steeper than that for aequorin.

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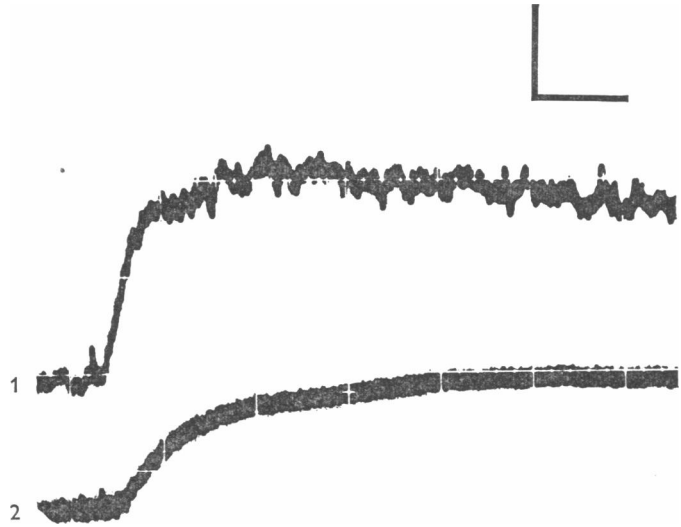

Fig. 1. Obelin light (trace 1) and tension (trace 2) responses from a bundle of barnacle myofibrils (diam. 95 μm) when activated with an externally applied calcium-EGTA buffer solution. The procedure is as described by Ashley, Moisescu & Rose (1974), Composition of solutions: high EGTA relaxing ($Ca^{2+} < 10^{-9}$ M) (mM) K_2-EGTA 20, KCl 40; low EGTA relaxing ($Ca^{2+} \sim 10^{-9}$ M) K_2-EGTA 0·1, KCl 80; contraction ($Ca^{2+} = 1{\cdot}2\ 10^{-6}$ M), total K_2-EGTA 20. All solutions contained in addition (mM) TES 10, Mg^{2+} 0·1, ATP (total) 4, pH 7·1 ± 0·01, temp. *ca.* 20° C. Calibration: vertical (1) 50 nA, (2) 0·25 mN; horizontal, 1 s. This result again suggests that, even at this low pCa, tension follows the changes in free calcium as was suggested at higher pCa values using aequorin (Ashley, Moisescu & Rose, 1974).

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A method for injecting aequorin into large muscle fibres using a micropipette

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The calcium-sensitive photoprotein aequorin was originally introduced into large *cannulated* barnacle muscle fibres (Ridgway & Ashley, 1967; Ashley & Ridgway, 1968) by a process of axial micro-injection (Hodgkin & Keynes, 1956). Here a method has been used which permits the introduction of aequorin into *intact* barnacle fibres by means of a glass micropipette inserted through the surface membrane of the muscle fibre. The glass micro-pipette is prepared conventionally and contains, at the tip, a glass 'fibre'. Small amounts of the aequorin solution (1 in 5 dilution of a

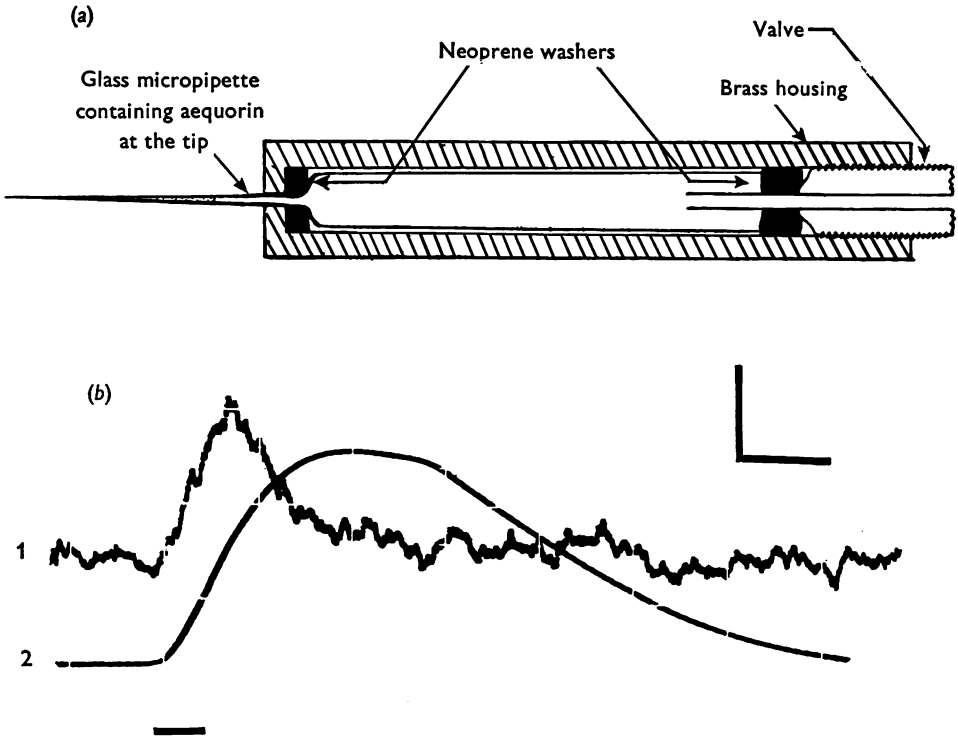

Fig. 1. (*a*) Schematic of the brass housing for holding the micro-pipette. (*b*) Light (trace 1) and tension (trace 2) responses from an isolated *intact* barnacle muscle fibre injected locally with aequorin through a micropipette. The horizontal line indicates the duration of the stimulus applied by Ag–AgCl plate electrodes. Calibration: vertical, (1) 20 nA; (2) 2 g wt. Horizontal: 200 msec. Temperature *ca.* 20° C. Fibre diameter *ca.* 1 mm. Stimulus intensity: 6 V. Time constant for trace 1, 40 msec.

saturated solution of aequorin in 100 mM TES buffer, pH 7·3) were introduced into the micro-pipette by means of a fine plastic capillary. A brass housing into which the micro-pipette was inserted (Fig. 1*a*) was connected directly to the output of an electro-magnetically operated flow valve (100 N cm^{-2} rating). The micro-pipette was sealed into the housing by two neoprene washers. Rectangular current pulses of 200–400 msec duration were used to activate the valve which was operated typically at 50 N cm^{-2} from a supply of compressed air. The external dimensions of the tip of the micro-pipette were intially 1 μM, but after penetration of the tough outer membrane of the muscle cell the final diameter was usually in the range of 2–3 μM. The first pressure pulse was always the most efficient at expelling the aequorin solution. Light emission responses were recorded by means of a 10 mM diameter photocathode photomultiplier tube (EMI 9502B) which was placed directly below the Perspex chamber containing the muscle fibre and opposite the region of injection. The fibres were stimulated either locally using a surface suction-electrode or preferably by employing full fibre-length plate electrodes. Typical light and tension responses from plate-electrode stimulation are illustrated in Fig. 1*b*. This preparation enables calcium and tension responses to be examined over a wider range of values than was possible with the cannulated preparation and this technique could also be used to investigate the effect of direct neural stimulation upon the aequorin-injected fibre.

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The use of aequorin and the isolated myofibrillar bundle preparation to investigate the effect of SR calcium releasing agents

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The myofibrillar bundle preparations isolated from barnacle muscle fibres has already been used to investigate the relationships between Ca^{2+} and tension (Ashley & Moisescu, 1973). Here the myofibrillar bundle preparation is employed in conjuction with the photo-protein aequorin to investigate the effect of agents known to release Ca^{2+} from internal sites, especially the sarcoplasmic reticulum (SR). Single barnacle muscle fibres were injected with a saturated solution of aequorin (see legend to Fig. 1)

and the fibres transferred immediately to a modified saline lacking calcium. The fibre remained in this saline for 5–7 hr in order to remove external calcium and to reduce the calcium concentration within the surface invaginations (clefts) to a low value. The fibre was removed and placed under paraffin oil and the bundles of myofibrils prepared and clamped for tension recording as described previously (Ashley & Moisescu, 1973). The aequorin light and tension responses of the myofibrillar preparation were examined for a period of 20–40 sec in a 'relaxing' solution

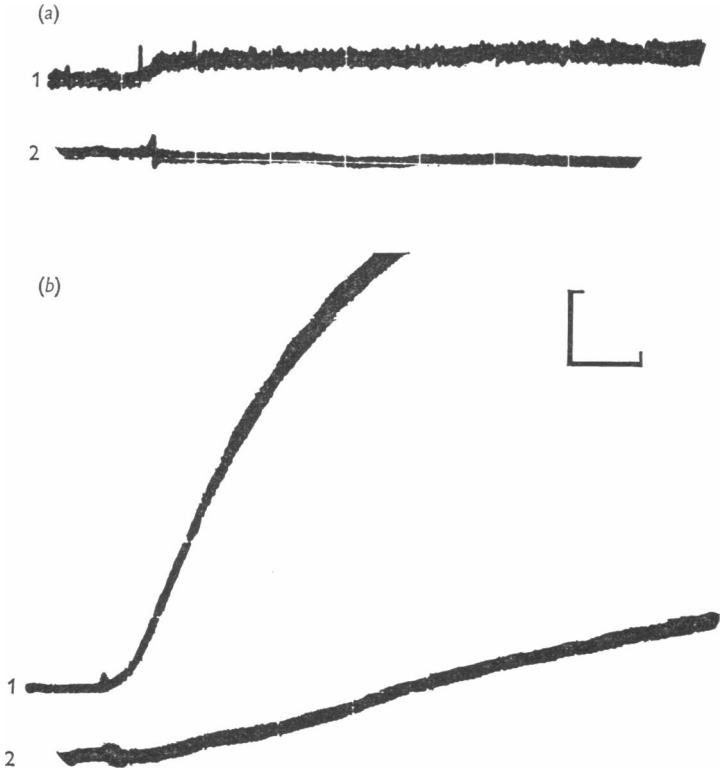

Fig. 1. Light (trace 1) and tension (trace 2) responses from a bundle of barnacle myofibrils isolated under paraffin oil (Ashley & Moisescu, 1973) from a cannulated single fibre loaded axially with 0·2 μl. of a saturated solution of aequorin in 100 mM-TES buffer, pH 7·3 and soaked in crab saline (Fatt & Katz, 1953) lacking calcium for 5 hr. (*a*) Bundle transferred directly to a 'relaxing' solution; (mM) K_2-EGTA 0·1, K-TES 130, ATP 4, Mg-acetate 1·15, sucrose 1000, pH 7·1 ± 0·01; and then moved into (*b*) a similar solution containing in addition 20 mM caffeine, temp. ~ 20° C, bundle diameter 200 μm. Calibration: vertical, (2) 0·15 mN (1) 200 nA in (*a*) and 1 μA in (*b*); horizontal, 2 sec. The 'blip' on trace 1 is electrostatic in nature and corresponds with the moment the preparation is immersed in the solution.

(see legend and Fig. 1*a*), before the preparation was transferred to 20 mM caffeine contained in relaxing solution. The light and tension responses rose immediately in the caffeine (Fig. 1*b* compared to 1*a*) indicating a rise in the Ca^{2+} in the myofibrillar bundle. Similar responses were observed when the preparation was immersed in a relaxing solution containing the non-ionic detergent Brij -36 (1·5 % w/v) or the ionophore A23187 (6 μg/ml.), although the responses in the ionophore were usually slower in onset. These results would be in agreement with the idea that the Ca^{2+} released by these agents is derived from the internal membrane system, particularly the SR. Certainly electron microscopical observations of myofibrillar bundles treated with Brij-36 indicates that this agent removes very effectively the internal membranes and hence would release any stored calcium. In separate but related experiments P. J. Griffiths (unpublished observations) has shown that single barnacle muscle fibres remain responsive to internal injections of caffeine after 5–6 hr in 0-Ca saline, particularly if 1 mM-La^{3+} is present externally to reduce the loss of internal calcium.

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Changes in extracellular fluid composition which excite axons

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Some solutions which cause pain when applied to skin may act directly on axons rather than on receptors, and there is evidence that a similar mechanism may operate in teeth (Horiuchi & Matthews, 1974). The object of the experiments demonstrated is to determine what changes in the composition of the extracellular fluid around axons will evoke a discharge of impulses. So far, the experiments have been limited to multifibre strands and to changes in the concentration of substances normally present in extracellular fluid.

Recordings are made from isolated spinal roots taken from the cauda equina of a rat (Evans, 1968). A small, anatomically distinct, bundle of fibres is separated from the cauda equina and set up in the bath shown diagrammatically in Fig. 1. Silicone grease is used to seal around the nerve between chambers. Differential recordings are made between this strand and a shorter one which passes only into the earthed chamber containing Krebs–Henseleit solution, an arrangement which provides a balanced recording system and an optimal signal to noise ratio. The part of the

nerve to which the test solutions are applied is normally bathed in Krebs–Henseleit solution gassed with 95% O_2 and 5% CO_2 at 37° C, having a pH of 7·4. Solutions are applied and removed with a syringe connected to the bottom of the test chamber. The test solutions are applied for

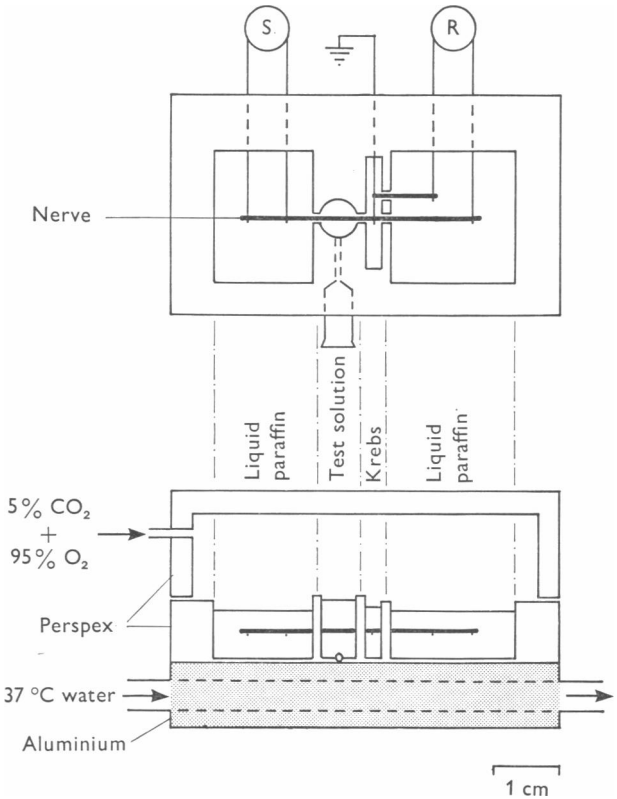

Fig. 1. Plan and sectional views of the nerve bath. The bath temperature is regulated by pumping water from a thermostatically controlled bath through the aluminium block. A three-way tap (not shown) placed in the female Luer connector at the base of the test chamber is used in the injection and aspiration of solutions. A moist atmosphere of 5% CO_2 and 95% O_2 is normally present over the nerve bath to maintain the partial pressure of these gases in the test and control solutions.

$1\frac{1}{2}$ minutes at 37° C and, with the exception of those in which the effects of CO_2 are investigated, gassed with 95% O_2 and 5% CO_2. Any tendency for CO_2 to be lost from the solutions is prevented by covering the bath with a lid enclosing an atmosphere of the equilibrating gas.

The effects of changing the concentration of Na^+, K^+, Ca^{2+}, Mg^{2+}, H^+ and CO_2 and of raising the osmotic pressure are being examined.

This work was supported by a grant from the M.R.C.

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Recordings from the terminals of intradental nerves

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Recordings with single unit resolution can be made from the terminal regions of some intradental nerves using relatively gross electrodes in contact with dentine (Horiuchi & Matthews, 1974). The slender, cone-shaped canine tooth of the cat provides a suitable preparation, and the success of the technique seems to depend upon the anatomical isolation of the dentine and pulp in the crown of the tooth which is covered by high resistance enamel.

The electrodes used have been developed to minimize noise and miscellaneous spikes of non-biological origin which proved troublesome in earlier experiments. Details of the method of construction of these electrodes are given elsewhere (Horiuchi & Matthews, 1974). Briefly, a close-fitting Perspex cap incorporating a compressed pellet of silver powder and silver chloride is cemented over the terminal half of the tooth and then a cavity is cut into the side of the cap to expose part of the Ag/AgCl and the tip of the tooth. The cavity is filled with Ringer solution and then enamel is carefully removed from the tip of the tooth with a slow-running dental burr. The Ringer solution provides electrical contact between the exposed dentine surface and the Ag/AgCl and is used in an attempt to maintain the composition of the extracellular fluid of the dentine near normal. Chemical stimuli can be applied to the same area of dentine by replacing the Ringer solution with some other electrolyte. Differential recordings are made between this electrode and an indifferent electrode in a large shallow cavity near the neck of the tooth.

In order to check the identity of spikes recorded from dentine and to record action potentials during antidromic invasion of the nerve terminals, strands containing single intradental fibres are dissected from nerves outside the tooth and set up for recording or stimulation. For this purpose it is easier to work on the lower canine tooth and the inferior dental nerve.

This work is supported by a grant from the Medical Research Council.

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Characterization of the giant cells in the abdominal and parietal ganglia of the giant snail *Archachatina*

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The nervous system of the gastropod mollusc has become a useful tool in the study of the neuronal control of behaviour. The giant cells in the ganglia play important roles in these patterns (e.g. the gill-withdrawal reflex of *Aplysia* (Kupfermann & Kandel, 1969); the control of circulation in *Aplysia* (Mayeri, Koester, Kupfermann, Liebeswar & Kandel, 1974); the escape reaction of *Tritonia* (Willows, Dorsett & Hoyle, 1973).

In order to investigate the functions of the giant cells in the ganglia of *Archachatina* the cells had first to be identified. In the adult snail (> 100 mm shell length) histological observations on the abdominal and right parietal ganglia demonstrate at least 100 cells per ganglion in the size range 100–500 μm. However, if the ganglia are examined under a dissecting microscope no more than 20–30 giant cells can be observed in each ganglion. During reconstruction of the ganglia an additional criterion was adopted, viz. only those cells containing nuclei of irregular outline (see Amoroso, Baxter, Chiquoine & Nisbet, 1964) were selected. By this method all the larger cells (20–30) were included but the majority of the 100 μm cells were excluded.

Ganglia were serially sectioned at 7 μm and every fourth section was photographed. The outlines of the giant cells, neuropil and emergent nerves were transferred to polystyrene tiles by means of a pantograph. Models were constructed of both adult and young animals. Although the sizes of the cells vary with the age (and therefore, the size) of the animal (see also Coggeshall, 1967, on *Aplysia*) it is possible to identify similar numbers of the largest cells in any animal. It is of interest that the 3-D reconstruction of giant cells shows that the irregular appearance of their nuclei is due to the outward spead of sheets of nuclear membrane and nuclear material through the karyoplasm as had earlier been suspected (Amoroso *et al.* 1964). Frequently a process of the nucleus is seen to extend into the neurite. Both of these features may be correlated with problems of nuclear control in such large cells.

From the models, plans were drawn of the distribution of the giant cells. It has been noted by Frazier, Kandel, Kupfermann, Waziri & Coggeshall (1967) and by Walker, Lambert, Woodruff & Kerkut (1970), working on *Aplysia* and *Helix* respectively, that the positions of giant cells vary from animal to animal. In addition, in no single preparation can all the known cells be identified: *Archachatina* is no exception. Thus, a giant cell cannot be identified merely from its position: its electrical activity must also be

investigated. Gayton, Kerkut, Lambert, Loker & Walker (1973) adopted a series of criteria to identify cells. We have found that a tactile stimulus to the collar is another useful way to differentiate between cells (Nisbet & Plummer, 1972, 1974). Correlation of each cell's position with its firing pattern has made possible the identification of approximately 20 cells in the abdominal and a further 20 in the right parietal ganglia.

A formalized presentation has been made of activity in a number of spontaneous cells, taken from lengthy pen recordings, in order to compare their activities when unstimulated. A corresponding plot of the same cells following peripheral stimulation has also been made. We have been unable to demonstrate any evidence of common interneurones controlling their activity. After peripheral stimulation, however, most cells are either excited or inhibited, suggesting that, directly or indirectly, they may be linked with the same interneurone pools that control the activity of the quiescent cells previously described (Nisbet & Plummer, 1972).

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A method of ligaturing the axons of molluscan neurones

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When recordings are made from the cell body of a molluscan neurone the properties of both the cell body itself and the axon with its associated synapses are observed. To permit a study of the cell body alone the axon can be ligatured near the cell body with a fine thread (e.g. Connor & Stevens, 1971) or a wire loop as demonstrated here.

A double-barrelled micro-pipette is pulled by hand from glass tubing 3 mm o.d. and 1·8 mm i.d. such that the final 7–8 mm consists of two

parallel tubes about 150 μm in diameter. At the other end one barrel must be left about 20 mm longer than the other to fit into the holder (see Fig. 1). The narrow part of the pipette is bent through an angle of about 30° using a de Fonbrune micro-forge and the end broken off to leave a distance of about 2 mm from the tip to the bend. If the glass does not break cleanly the tip must be ground smooth on a fine stone and fire polished.

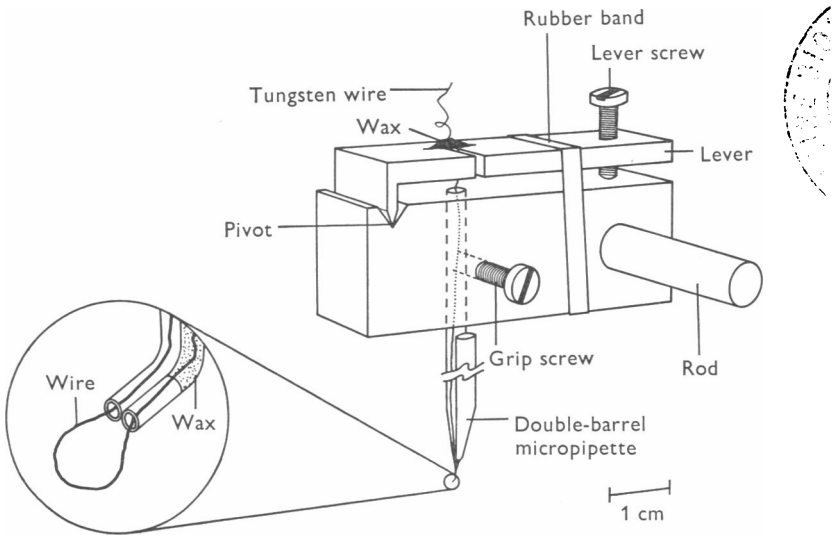

Fig. 1. Diagram of the apparatus, approximately to scale except for the inset. Parts are plastic unless otherwise stated.

A tungsten wire 13·5 μm in diameter (Goodfellow Metals Ltd, Esher, Surrey) is threaded up the long barrel of the pipette from the tip until it protrudes from the opposite end. The other end of the wire is pushed into the short barrel to form the loop and secured there by melting a few pieces of dental sticky wax that have been dropped into the pipette. The pipette is then mounted in the holder as shown in Fig. 1. The free end of the wire is pulled through the slit in the lever, until the diameter of the loop is about 0·5 mm, and fastened to the lever with sticky wax. The lever can be raised by the lever screw, thus pulling on the wire and tightening the loop.

In use, the holder is attached to a micromanipulator and the loop is passed over a group of cell bodies in an isolated and dissected molluscan ganglion. The loop is then closed around the axons and the device gently raised until the axons break and the cell bodies are completely detached from the ganglion. The best results were obtained by isolating two cells at a time; single cells were always badly damaged because the ligature had to be made very close to the cell body.

After an experiment the lever is lowered and the loop can be reopened with a pin and used again.

I wish to thank Professor F. Strumwasser for suggesting the method. The work was supported by a fellowship in the European Science Exchange Programme awarded jointly by the Royal Society and the Centre Nationale de Recherche Scientifique.

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A floating current clamp for intracellular injection of salts by interbarrel iontophoresis

BY R. C. THOMAS. *Department of Physiology, University of Bristol, BS8 1TD*

Quantitative injections of salts into single neurones are best made by passing a current between two intracellular micro-electrodes. To avoid artifacts it is important to have no current flow to earth, so the current source must be 'floating', and must also have minimal capacitance to earth. Since micro-electrode resistances change, it is helpful to have a system that will pass a constant current through varying resistances. The circuit shown in Fig. 1 will pass constant currents of 1–100 nA in either direction through resistances of up to 100 MΩ (or smaller currents through larger resistances) with no current flow to earth. Its response time is kept relatively long to reduce interference, so it is unsuitable for stimuli shorter than 1 sec.

The current-source part of the circuit was inspired by two early designs: for a light-coupled pulse generator (Waud, 1967) and for a stimulus isolator (Baird, 1967). The basic idea is that the current flowing through the micro-electrodes is measured as the voltage drop across a 1·0 MΩ resistor. The difference between this voltage and that set by a d.c. voltage source is amplified by a factor of 10^5 and used to activate one of the light-coupled isolators, thus controlling the voltage applied to the micro-electrodes. Since about 2 V are required to activate the light-emitting diodes, the minimum error is about 0·02 mV, or 0·02 nA. (Since blockage of a micro-electrode could apply most of the battery potential to the unity-gain amplifiers, and thus to both inputs of the high-gain amplifier, it is important that the latter has a high common-mode rejection ratio, and that the battery voltage be kept below 15 V.)

The circuit is designed so that before use as a current clamp one of the unity-gain followers can be used in conjunction with the triangle generator to measure electrode resistances (Strickholm & Winston, 1969), and then

to record potentials as the micro-electrodes are inserted into the selected neurone. During this, the clamp is held inactive by disconnecting the output from the high-gain amplifier, using switch S_2. Before use, the amplifier is reconnected, S_3 closed and R adjusted to give zero amplifier output.

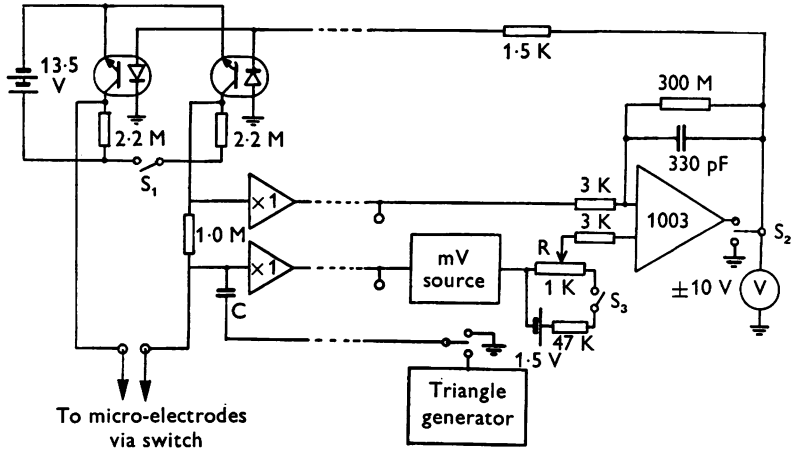

Fig. 1. Circuit diagram of the floating current clamp. The 13·5 V battery is made from ten RM 312 batteries clamped together; the light-coupled isolators are Texas Instruments TIL 107; the unity-gain amplifiers are integrated circuit F.E.T. operational amplifiers – Analog Devices AD 503; the high-gain amplifier is a Philbrick-Nexus 1003. Resistors are all 10% except the 1·0 MΩ, which is 1%. The 330 pF capacitor is polystyrene, and C is an air-gap formed by two turns of insulated wire round the amplifier input.

With S_1 closed, the desired current can then be made to pass (if the micro-electrodes will carry it) by applying the appropriate voltage from the mV source. (If the output voltmeter then goes off-scale, it indicates electrode blockage.) A three-way six-position switch (not shown) is used so that the clamp output can be applied between different micro-electrodes, or between one micro-electrode and earth. It is important that normally there be no voltage differences between the two current electrodes, or a current will flow when S_1 is closed. This can be checked by measuring the voltage across the 1·0 MΩ resistor when closing S_1.

The circuit is built in two parts: the left-hand part of Fig. 1 is assembled in a plastic block (100 × 50 × 12 mm) and the right-hand part is built on a panel outside the screened experimental enclosure.

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A twelve-way rotary tap for changing physiological solutions

BY L. DONALD PARTRIDGE* and R. C. THOMAS. *Department of Physiology, University of Bristol, BS8 1TD*

The problems involved in changing solutions flowing in a small recording bath have been dealt with in many ways (see, for example, Hodgkin & Horowicz, 1959; Holder & Sattelle, 1972). We demonstrated a 12-way tap of fairly simple construction which allows changes to be made between various bathing solutions easily and with minimal disturbance. Its main advantages are: simplicity of operation, large number of channels, almost no flow interruption, no solution wastage and minimal dead space. With the dimensions shown, it is suitable for flow rates of up to several ml./min.

The tap is illustrated in Fig. 1. Solutions flow into the tap through up to 12 polyethylene tubes, only two of which are shown in the figure, from storage bottles above. The input tubes fit into holes in the stator, arranged at 30° intervals in a circle round the central output hole. A 1·2 mm diameter U-shaped hole in the rotor connects the desired inflow tube to the central outflow. Since little force is required to operate it, the tap can be mounted close to the experimental bath, thus minimizing the delay before a new solution reaches the bath. The shaft can be made long so that the handle can be outside any screening.

The bearing plate, rotor and stator are made from 10 mm PTFE, to minimize friction, while the driver, shaft and handle are made from Perspex. The 4 mm stainless-steel plate provides stiffness, but is not essential. Each of the 13 holes in the stator are countersunk to accept a 3·5 mm diameter O-ring to give a watertight seal between these holes and the (carefully polished) face of the rotor. The polyethylene tubes (we use Portex PP 90) pass through clearance holes in the steel plate and are held by push fits into the (tapering) holes in the stator. The whole assembly is held together by two bolts between the bearing plate and steel plate.

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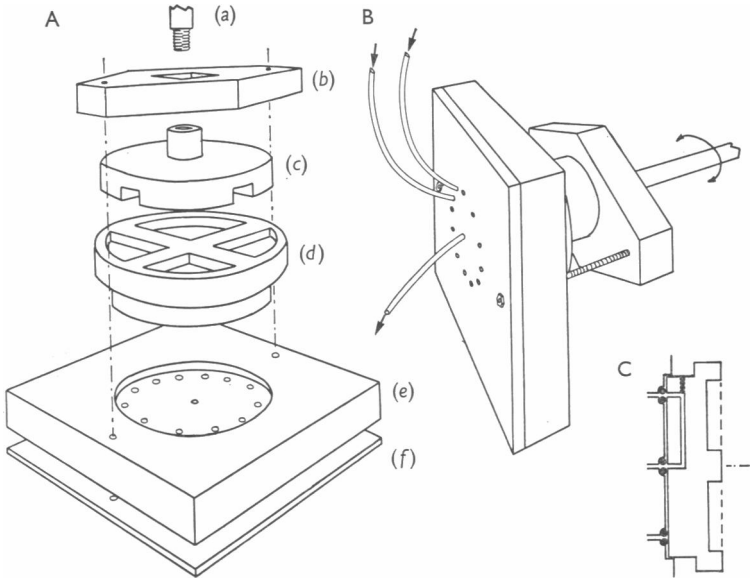

Fig. 1. Diagrams (not to scale) showing construction of 12-way tap. (A) Expanded view of the main parts: a PTFE washer between (*b*) and (*c*) and the O rings set into (*e*) are not shown. (B) The tap assembled, showing two input tubes and the central output. (C) Transverse section through the rotor and adjacent face of the stator to show the channel connecting selected input to the central output, and the position of the O rings on the stator. The main dimensions of the parts (in mm) are as follows. Shaft (*a*) rod diameter 9·5. Bearing plate (*b*) 58 × 38, with central hole 16 × 16. Driver (*c*) diameter, 25, height 18. Rotor (*d*) upper diameter 36, lower diameter 28. Stator (*e*) 51 × 38, central recess diameter 28, depth 2. Stainless-steel plate (*f*) also 51 × 58.

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A simple micromanipulator for investigation of cerebellar neurones in unrestrained cats

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We are currently studying the firing patterns of individual cerebellar neurones in unrestrained cats. It has been our aim to minimize the surgical interference required to allow access to the cerebellum and we have therefore chosen an approach between the bony tentorium and the lambdoidal

* M.R.C. Research Scholar.

ridge. The largest craniotomy possible in this region is less than 1 cm in diameter so that we have been led to design and construct a micro-manipulator which is both small enough to affix over the craniotomy and light enough not to interfere with the normal behaviour of the animal.

The micromanipulator is shown in Fig. 1. It consists essentially of a stainless-steel rod (1) threaded at 90 T.P.I. and passing through a fixed collar (2). The rod carries two fixed washers which work against the collar; it moves an internally threaded nylon piston (3) along a bore cut in the cylindrical body of the manipulator and a key (4) prevents rotation of the

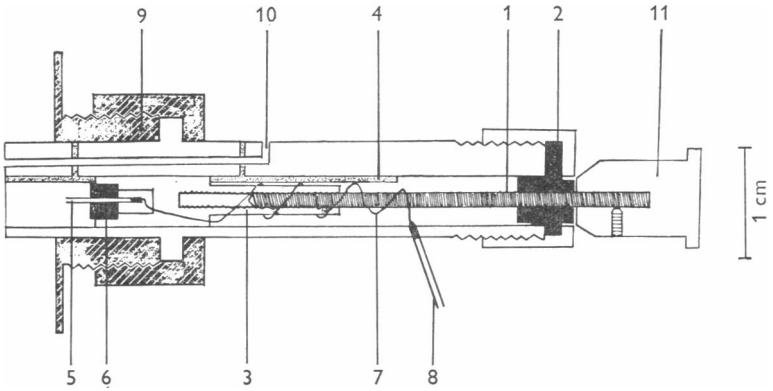

Fig. 1. Manipulator and titanium chamber in cross-section from the side. 10, Port to relieve pressure within the chamber. 11, Control knob. Other numbers are explained in text.

piston. The body is made from polypropylene for strength, resilience and ease of sterilization. The manipulator is used in conjunction with a tungsten-in-glass micro-electrode, manufactured by a variant of the process described by Merrill & Ainsworth (1972). The electrode is push-fitted into a piece of stainless-steel hypodermic tubing (5) embedded in a boss (6) which is in turn embedded in the piston head. Braided stainless-steel cable (7) is used to make electrical connexion between the micro-electrode and a gold-plated socket (8) on the exterior of the manipulator. During recording sessions the manipulator is fitted into the top of a titanium chamber (9) implanted stereotaxically over the craniotomy. Weight, including the chamber and the dental acrylic used to lock chamber to skull, is only 14 g.

Variations in the point at which the micro-electrode penetrates the dura are achieved by an eccentric positioning of the bore within the body of the manipulator and by rotating the body within the titanium chamber. The degree of eccentricity of the bore determines the radius of the circle described over the brain surface of the micro-electrode tip. We routinely

use two manipulators, one with radius 2·5 mm, the second with radius 3·5 mm.

Disadvantages of the instrument include some backlash, the difficulty of measuring depth of micro-electrode penetration (by counting turns; 260 μm per turn) and the experimenter–animal interactions which are inevitable during use. For our application these difficulties are outweighed by numerous advantages which include lightness, small size, cheapness, simplicity of construction, ease of sterilization (by autoclave or by alcoholic sterilants) and the large range of linear movement which can easily be provided (20 mm in the current models).

The demonstration showed one manipulator disassembled for servicing and one in the recording position.

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The Modular One computer system in the Department of Physiology

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The Department was awarded a grant by the Medical Research Council to develop a multi-access laboratory computer system to provide in each of a number of laboratories facilities comparable with those of a PDP-12 or LINC-8. The Modular One computer manufactured by Computer Technology Ltd (C.T.L.) was chosen for this development because of a number of features of its hardware design which make it especially suitable for this sort of work.

The first part of the system was delivered in June 1969. This did not include any analogue hardware, so the machine could not be used on-line to an experimental set-up. However, its arrival did give us the opportunity to get used to operating and programming the machine and to begin to develop the software necessary to facilitate its use in a laboratory environment. The most important software to be produced was an editing and operating system which operated interactively via teletype and display. This was based on the general philosophy of the LAP-6 system for the LINC computer, and soon reached the stage where it had sufficient facilities and was sufficiently reliable to be used to operate upon and modify itself. After this, software development became considerably easier and quicker, although the lack of any backing store – such as the magnetic tapes on a LINC-8 – was a hindrance, since all programming involved the use of a

great deal of paper tape. We also wrote a number of other items of software, of which the most generally useful were a series of routines for performing floating point arithmetic operations.

By the middle of 1970 we had begun to run experiments on-line to the machine on a one-at-a-time basis. In this way, we were able to get a large amount of useful work done, even though the computer was not operating as a multi-access system. In our initial planning of the development, the proposed method of implementing a multi-access system had been based on a special interface unit and a general-purpose laboratory executive, both of which C.T.L. had committed themselves to produce. However, by mid-1970 it was clear that it would be some considerable time, at least, before either of these items were provided, and indeed, neither has appeared even now. We therefore decided to adopt a different approach, using hardware that was actually available and developing a suitable executive. The Medical Research Council provided additional help in the form of programming assistance to develop the executive and further hardware, including a magnetic disk to act as backing store. After some difficulties, our editing and operating system was modified so that all data could be stored on the disk. In this way the use of paper tape (except as a permanent form of storage) has been almost completely eliminated, making programming and operating the machine much quicker and easier.

Despite the Modular One's excellent hardware, the development of a multi-access system has taken considerably longer and more effort than was originally hoped. The main factors involved have been the non-appearance of the special interface originally ordered and the C.T.L.s inability to provide suitable software. However, the machine has been used for a great deal of useful experimental work. The multi-access executive is now operational and was fully described in the following Demonstration (Wyon, 1975). The system was demonstrated performing interactive tasks simultaneously with the processing of data from two experiments (Armstrong, Cogdell & Harvey 1975; Lewis & Luff, 1975).

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The MAFIA Executive for the Modular One

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The Modular One Computer in the Physiology Department at Bristol is at present used by four experimental groups. The computer is in a central room and is accessed remotely from teletypes, which can be moved where required. The teletype and data relevant to each experiment are connected to the computer by cables of up to 50 m in length.

Until recently, all programs have been run under an executive program supplied by Computer Technology Ltd (C.T.L.), called E 2. Only one experimental program at a time can run satisfactorily under this executive, the main reason being the need for the processor to be fully dedicated to data acquisition when this is occurring at rates above 500 Hz because of the slow response of E 2. This limitation could be removed by acquiring data either by direct access to store or by using interrupts to inform the processor when a data sample is necessary. In the absence of suitable hardware for the former solution, the latter option was left.

The problem was to find an executive which would allow experiments to be carried out simultaneously, with data acquired by interrupts. All executives available for the Modular One were considered and rejected due to failure to meet one or more of the departmental requirements. The MAFIA (Multi Access Fast Interrupt Acceptance) executive has been designed with the following objectives:

(1) Multi-access. With experiments situated in Laboratories up to 100 m apart the concept of a single 'control' or 'executive' teletype is undesirable. Each user should be unaware of other users and appear to have full control of the system.

(2) Fast interrupt response. With data read in response to interrupts, this should be as fast as possible since sampling rates of 5–10 kHz are used. A target of 100 μsec maximum response was set, which would allow a 10 kHz total data rate for the system.

(3) Compatibility with E 2. This is desirable not only to minimize changes to programs written in the department, but also to allow continued use to be made of other programs written for E 2, including standard programs supplied by C.T.L.

(4) Reasonable size. Some of the experimental programs are now quite large, and it was felt that any appreciable increase over the 6 K words of store occupied by E 2 might prove embarrassing in a multi-access system working in a total of 24 K words.

The MAFIA executive has now been developed to the point where it can be used for experiments. It has achieved all of the above aims and has

been designed to allow the later addition of more sophisticated facilities if desired. The interrupt response is 85 μsec. It is compatible with E 2 in all but a few trivial cases, and offers more facilities and flexibility. The size of MAFIA is at present about 4 K words, including a disk filing system, with a variable amount of workspace dependent upon the number of users, programs/user, devices, etc. For a typical system with three users this would be about 3 K words.

Special features include consistent handling of all input and output devices as far as user programs are concerned, communication between programs via message channels and handlers to facilitate analogue input and output.

A hardware interrupt controller has been constructed which allows interrupts into the machine from up to ten different sources in blocks of up to 2048, with the clock rate and start time under user control and a block size which is set by the program.

Responses of interpositus neurones to stimulation of forelimb cutaneous afferents in chloralose-anaesthetized cats

By D. M. Armstrong, Barbara Cogdell* and R. J. Harvey. *Department of Physiology, The Medical School, University of Bristol, Bristol BS8 1TD*

The main output from the cerebellum is provided by the axons of the intracerebellar nuclear neurones. It is therefore of considerable interest to study the firing patterns of these neurones under well-defined input conditions. We are currently studying the responses evoked in cells of nucleus interpositus in the cat by electrical stimulation of forelimb cutaneous afferents. Extracellular recordings are made using glass micropipettes filled with 4 M-NaCl (impedance 2–5 MΩ at 1000 Hz) and interpositus neurones are antidromically identified by stimulation of their axons in the region of the red nucleus. Unit discharges are recorded on magnetic tape and are also analysed on- and off-line using the Modular One computer system.

Animals are being studied using chloralose anaesthesia because there is good evidence that information processing within the cerebellum is powerfully affected by barbiturate anaesthetics (Gordon, Rubia & Strata, 1973). The demonstration animal is anaesthetized with 50 mg/kg of recrystallized α-chloralose and paralysed with gallamine. We have found that a cutaneous afferent volley evokes short latency accelerations and decelerations of the resting discharge which have previously been noted in decerebrate and barbiturate-anaesthetized animals (Eccles, Rosén, Scheid & Táboříková, 1972). However, there is present also in many neurones a powerful burst

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of impulses which typically begins with a latency of 100–120 msec and lasts from 100 to 400 msec. This burst is abolished by as little as 5 mg/kg of pentobarbitone.

These responses were displayed by using the computer to construct post-stimulus histograms and to display them on a screen. The time base of the display and the number of responses summed are both variable, and appropriate values are chosen using the teletype keyboard.

The late burst of impulses occurring in interpositus neurones will lead to a powerful facilitation of red nucleus neurones via the interpositorubral axons. It therefore provides an explanation for very similar bursts evoked from rubro-spinal cells by cutaneous afferent stimulation in chloralose-anaesthetized and unanaesthetized paralysed cats (e.g. Massion & Albe-Fessard, 1963). Investigations are in progress to determine whether this response is present in unanaesthetized unrestrained cats.

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On-line computation in iontophoretic studies of single units in the rat caudate nucleus

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These experiments have been designed to investigate and compare the behaviour of cells in the caudate nucleus of the adult and 21-day-old rat. The responses of cells to the iontophoretic application of various pharmacological agents and also to synaptic stimulation are being studied. Using a Linc-8 computer (D.E.C.) a system has been developed to control and monitor drug application and to provide a 'bank' of programs which can be selectively called in when required to provide 'on-line' analysis of cell firing patterns.

Recordings are obtained using seven barrel micro-electrodes (tip size 1·0–3·0 μm, recording barrel filled with 3 M-NaCl and a.c. resistance at 25 kHz, 15–25 MΩ in saline) stereotaxically placed in the caudate nucleus of a DIAL anaesthetized rat (1·0 ml/kg I.P. adult, 0·25 ml./kg I.P. 21-day). Cell activity is simultaneously recorded on magnetic tape using an analogue tape-recorder and fed directly into the computer. Ejection of drugs from

* S.R.C. Scholar.

the six outer barrels is controlled by the current-passing device (modified from Moss & Beechey, 1972), which can be manipulated either manually or by the computer. Synaptic stimulation via the sciatic nerve, motor cortex or in the substantia nigra is monitored and recorded, as are iontophoretic currents.

In this demonstration the use of two programs was shown. 'DRUGSY' enables the experimenter to set the duration and sequence of drug application and can control up to six channels simultaneously. During drug application a frequency histogram of cell activity is prepared and this can be displayed on completion for photographing or drawn out using a graph-plotting subroutine.

A second program-suite 'MULTI' makes available interval histograms, autocorrelograms, cross-correlograms where two units are recorded at the same time, and also a program for statistically comparing any two selected sets of data; e.g. with and without drug application, or electrical stimulation, or the behaviour of two different cells. All the displays may be photographed or plotted out.

These programs are designed to characterize the firing patterns of the cells studied and also to attempt to define the relationship, if any, between two cells when they are recorded simultaneously. It is probable that interaction between caudate cells is important in their functional organization since it has been found that the neuronal population consists mainly (about 95%) of one type of neurone (Kemp & Powell, 1971), a medium-sized short axonal interneurone with spiny dendrites. Thus, the predominance of interneurones would indicate that establishing the caudate nucleus as a functional entity depends largely on intra-caudate relationships, which may well define the behaviour of the caudate nucleus in its role in the control of movement.

The advantage of using 'on-line' analysis of cell activity is that concurrently with conducting the experiment it is possible to vary the parameters of the experiment in relation to the observed effect, thus modifying the design of the experiment itself according to the analysed results.

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The contraction of dually innervated muscle fibres in the rat

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Extrafusal fibres of adult mammalian skeletal muscles normally are innervated by single axons; however, Jansen, Lømo, Nicolaysen & Westgaard (1973) have demonstrated that it is possible to induce polyneuronal innervation by special procedures. It is known that the contractile properties of muscles are influenced by their innervation (Buller, Eccles & Eccles, 1960) and it may be possible to gain information about the mechanism of operation of this influence by studying the contractile properties of dually innervated fibres. We have adapted the method of Jansen *et al.* (1973) which results in fibres of soleus muscle (slow twitch) being re-innervated by their own nerve and also by a nerve which had originally innervated a fast twitch muscle.

Analysis of the responses of these dually innervated fibres would need to take into account two major factors. First, cross-reinnervated muscle does not necessarily take up all the properties of the muscle from which the nerve was derived. For example, a slow muscle innervated by the nerve from a fast muscle has an intermediate twitch speed (Close, 1969). Secondly, the contractile properties of a muscle can be influenced by the activity of synergists and antagonists (e.g. Gutmann, Shiaffino & Hanzlinkova, 1971). In the present experiments the innervation of antagonists must necessarily be damaged in obtaining the fast muscle nerve for dual innervation of soleus. For these reasons it is important to be able to compare the contractions of dually innervated fibres with those of cross and self re-innervated fibres within the same muscle. In such a preparation the recorded contractions will be those of more than one group of fibres and the purpose of the demonstration was to show how we have attempted to analyse the complex responses.

The operative techniques were derived from those of Frank, Jansen, Lømo & Westgaard (1973). In 8-week-old rats anaesthetized with sodium pentobarbitone, the nerve to either extensor digitorum longus or the peroneal muscles (all fast twitch) was freed and tied to the surface fascia of soleus muscle. Two to three weeks later the animals were anaesthetized again and the nerve to soleus was cut near the muscle. These procedures allow some muscle fibres to become dually re-innervated by axons from both nerves, whilst others receive axons from only one of the two nerves. Isometric contractions were recorded 8–12 weeks later, after preparing the two nerves for stimulation.

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The isometric contractions (as a function of time) will be represented symbolically as $f(\,)$. The experimentally recorded contractions are distinguished by capital letters: $f(\mathrm{F})$, $f(\mathrm{S})$ and $f(\mathrm{F}+\mathrm{S})$ to represent respectively the responses to stimulation of the fast muscle nerve alone, soleus nerve alone and both nerves together. The theoretical contractions of those fibres innervated only by the fast muscle nerve will be symbolized as $f(\mathrm{f})$, those innervated only by soleus nerve as $f(\mathrm{s})$ and the dually innervated fibres as $f(\mathrm{fs})$. If the contractions of the fibres sum linearly, then the two sets of responses will be related according to three equations:

$$f(\mathrm{F}) = f(\mathrm{f})+f(\mathrm{fs}), \quad f(\mathrm{S}) = f(\mathrm{s})+f(\mathrm{fs}),$$

$$f(\mathrm{F}+\mathrm{S}) = f(\mathrm{f})+f(\mathrm{s})+f(\mathrm{fs}).$$

In the case of isometric tetani in which constant tension is reached, solution of the equations is simple as $f(\,)$ may be represented by the maximum tension (cf. Bagust, Lewis & Westerman, 1973). For the twitches the full time courses of the responses must be used. A digital computer program has been developed which allows several responses to be averaged (see Bagust *et al.* 1973), but this facility has been modified so that responses can be subtracted as well as summed. This program may be used to estimate the contraction of the dually innervated fibres as:

$$f(\mathrm{fs}) = f(\mathrm{F})+f(\mathrm{S})-f(\mathrm{F}+\mathrm{S}).$$

Other facilities of this program were demonstrated as part of a multi-access system (Harvey, Williams & Wyon, 1974).

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Densitometry of scanning electron micrographs in the elucidation of chromosome structure

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With the light microscope chromosomes have been seen for nearly a century to have a helical structure (Baranetzky, 1880; Manton, 1941), evidence for which is obtainable with living animal cells (Boss, 1959). Nevertheless, transmission electron microscopy of isolated chromosomes has shown not a spiral but an irregularly packed chromatin thread (Du Praw, 1970). The question arises whether the conflict of evidence is attributable to differences in method of preparation and, in particular, to the use of hypotonic solutions and distilled water in the isolation of the chromosomes for electron microscopy.

By repeated pipetting cultivated human (HeLa) and Chinese hamster (DON) cells were sheared and their contents released (Pawlowitzki & Blaschke, 1971). The material was then coated and examined by scanning electron microscopy. A hypotonic solution and trypsin were also used for isolation, and the use of these was varied in an attempt to obtain 'clean' chromosomes. No procedure was found that did not either destroy (or possibly collapse) the chromosomes or leave them with a coating of apparently cytoplasmic origin. (A full description of the series of procedures is given by Hatami-Monazah, 1974.) The coated chromosomes appeared sometimes to be ridged and grooved, but direct measurements could not be precise and subjective impressions could have been misleading.

Transparent prints of micrographs of the surface of chromosomes were therefore analysed by a 'Photoscan' densitometer by which, from contiguous circular areas 0·1 mm in diameter, densities were recorded according to a scale of 255 shades of grey. Each primary record was on magnetic tape, from a computer print-out of which the values of densities were set out in an array which was thus a density map of the area scanned. From each array several lines of density values, parallel with each other and roughly parallel with the long axis of the chromosome, were taken, and density was plotted against distance for each line. Also, readings at the same distance along parallel lines were summed, i.e. the rows of numbers were placed under each other and added vertically. In this way features common to the rows could be largely cleared of noise and transversely or obliquely running lines of high and low density be made more evident.

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In each of 10 chromosomes one area was scanned, 8 of these chromosomes showing possible ridging and grooving on direct inspection of the micrographs. In these 8 areas regularity of transverse or oblique banding was confirmed by analysis of the densitometric scans, the cycle periods being constant over most of any one area and ranging, in the series, from 0·1 to 0·7 μm. Thus there were periodicities of the same order of size as those of helices seen by the light microscope.

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The use of variability in nuclear DNA content in characterizing a cell population

BY J. M. N. BOSS and R. C. L. FENELEY. *Department of Physiology, Medical School, Bristol BS8 1TD, and Bristol United Hospitals*

A cell may be characterized by the DNA content of its nucleus, and variations between the DNA complements of cells are often demonstrated by differences in karyotype. However, it is simpler, and sometimes sufficient, to measure the DNA content of each of a sample of cells in a population and make some measure of the variability of values. This approach is suitable for a tissue which may have gross differences from cell to cell in chromosome number.

In the example presented in this demonstration the tissue is human vesical uro-epithelium, taken during life by transurethral resection; 19 samples were taken from 15 distinct sites in 14 subjects. The epithelium was smeared immediately after removal, fixed in 10% lanthanum acetate in formol saline and stained by the Feulgen reaction (6 min hydrolysis). The integrated absorption of a nucleus was measured with a Vickers M85 microdensitometer in the band 540–580 nm and taken as proportional to the DNA content. The $\times 42$ lens had a N.A. 0·95, and the scanning spot was 0·5 μm in diameter. In each smear measurements were made on 25 nuclei. For each 25 the mean and standard deviation (expressed as a fraction of the mean) were calculated, and the latter taken as one measure

of variability. A second measure was obtained by making a rankit plot of the 25 values, and fitting a straight line to give the fewest points more than $0{\cdot}25 \times$ standard deviation from itself. (In this case 'standard deviation' means the parameter σ graphically determined by the fitted line itself and not a S.D. calculated from all readings.) The number of points (here called 'deviants') more than $0{\cdot}25\sigma$ from the fitted line were then counted. The number of 'deviants' thus provides another measure of variation.

Tumours from which epithelium was taken had been independently classified histologically into 'low', 'average' or 'high' grades (Miller, Mitchell & Brown, 1969), and the samples examined were 7 normal, 3 from 'low' grade tumours, 6 from 'average' and 3 from 'high'. All normal and low had S.D. $< 0{\cdot}4$ and deviants $\leqslant 8$, while all 'high' had S.D. $\geqslant 0{\cdot}6$ and such deviation in the rankit plots that no line could be drawn. The 'average' grade tumours yielded smears with S.D. $= 0{\cdot}2$–$0{\cdot}6$ and 'deviants' numbering from 2 to an indefinitely large number, as for those classified 'high'.

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The mode of packing of chromatin in human fibroblasts

BY J. M. BOSS. *Department of Physiology, Medical School, Bristol BS8 1TD*

Previously Boss, Hollis & Mysliwski (1973*a*, *b*) reported methods for measuring the inhomogeneity of distribution of chromatin in interphase human pulmonary fibroblasts in cell culture. Data were obtained with a Zeiss 'Cytoscan' scanning microdensitometer, using Feulgen preparations of the cells, and the data from each nucleus included the area, the integrated absorption ($\equiv$ total desoxyribonucleic acid), and the areas with absorption values between optical density 0·01 and 0·02, 0·02 and 0·03, etc. This demonstration showed a re-working of those data with Tchacarof's index of inhomogeneity (Tchacarof, Natchev, Mitrami & Boev, 1965). This is given by

$$K = \frac{1}{\log A} \sum_{n=1}^{A} p_n \log p_n,$$

where K is the index, A is the number of density classes, and p is the fraction of the area in the class n. (The equation is that used to find the entropy increase when A separate things, each being p_n of the total, are homogenized together.)

For each of 66 nuclei $A = 10$ and p_n relates to a range of optical density

of 0·03, the range of optical densities thus considered being therefore 0–0·3. (No density reading greater than 0·3 was among the data.) The integrated absorption indicated whether the DNA had or had not replicated, and there was no evidence for a relationship between Tchacarof's index and the stage of the life cycle. However, the index was related to the mean density of the whole nucleus ($r = 0{\cdot}8858$ for 66 pairs of values). For each nucleus the individual density readings (each for an area 0·5 μm diam.) were then taken in rank order, and the area of the nucleus divided into the densest quarter, second densest quarter, third densest quarter and least dense quarter. The mean densities of the densest quarters were then plotted against the mean densities of the whole nuclei, as were the mean densities of each of the other three quarters. Over a wide range of densities there was apparent linear proportionality for all four quarters. This suggests that a denser nucleus is less homogeneous because all densities are higher by the same proportion, but therefore not by the same absolute amount.

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Experimental myelination in dystrophic mice

By C. A. Stirling. *Department of Physiology, University of Bristol, BS8 1TD*

An experimental approach for inducing myelination of amyelinated nerve fibres in dystrophic mice is being used to investigate Schwann cell–nerve fibre interactions. Dorsal roots and sciatic nerves are being crushed and recovery investigated with light and electron microscopy. Animals and samples of nerves examined were shown.

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Recording from nerve cells or muscle grown in tissue culture

By S. N. Lawson. *Department of Physiology, University of Bristol*

Trypsin dissociated rat and mouse nerve cells and myoblasts have been grown in culture by a number of methods, most recently on Aclar strips or collagen coated cover-slips in roller tubes and in Petri dishes. Cultures were shown and also a method for recording the membrane potentials and

electrical responses of nerve cells or muscle. For this procedure cultures are placed on the stage of an inverted microscope and viewed at magnifications up to $\times 400$ with phase contrast optics. Conventional microelectrodes are used with appropriate electronic stimulating and recording equipment, and electrode placements are made under direct vision.

Supported by a grant from the Medical Research Council.

The use of the mouse in neurophysiological experiments

By T. J. Biscoe, P. M. Headley and M. Martin. *Department of Physiology, University of Bristol, BS8 1TD*

The demonstration showed how we carry out electrophysiological experiments on the spinal cord of the mouse. The mouse is being used because of our interest in the physiological effects of the lesions to be found in the C.N.S. of behavioural mutants. The experimental arrangements allow stimulation of dorsal or ventral spinal roots or sciatic nerve and recording with single or multibarrel micro-electrodes. So far we have been able to record most of the usual electrophysiological phenomena seen in the spinal cord of other species including Renshaw cell activity, dorsal root reflexes and responses to electrophoretically applied drugs (excitation with DL-homocysteate or L-glutamate and depression with glycine and γ-aminobutyric acid).

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Investigations of the lesions in the nervous system found in some behavioural mutants of the mouse

By T. J. Biscoe, K. W. T. Caddy, D. J. Pallot, U. M. M. Pehrson and C. A. Stirling. *Department of Physiology, Bristol University, BS8 1TD*

The methods used in these investigations are those of light microscopy, electron microscopy, autoradiography and electrophysiology. The electrophysiological methods are demonstrated by Biscoe, Headley & Martin (1975) at this meeting. In this demonstration some of the mutants on which we are currently working were shown together with examples of the structure of the lesions present. These include the absence of Schwann cells in spinal roots and cranial nerves of the dystrophic mutant (Bradley & Jenkinson, 1973; Salafsky & Stirling, 1973; Stirling, 1975; Biscoe, Caddy, Pallot, Pehrson & Stirling, 1974), the lack of myelination in the Jimpy mutant (Sidman, Dickie & Appel, 1964) and the lesion in Lurcher.

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A method for recording with minimal artifact the e.e.g. of unanaesthetized, newly hatched chicks

BY G. HORN and B. J. MCCABE. *Department of Anatomy, University of Bristol, BS8 1TD*

Several workers have recorded the e.e.g. from unanaesthetized chicks by connecting the electrodes directly to the recording amplifiers (Key & Marley, 1961; Spooner, 1965). These techniques are suitable for recording from quiet chicks but are not satisfactory for recording from chicks which move more energetically, as in a training wheel, because of artifacts which result from movement of the cable. A technique was therefore developed in order to record the e.e.g. free from such artifacts. Briefly, a pair of intracerebral electrodes are connected to Field Effect Transistors (FETs) fixed on the animal's back, the FETs being used as source followers.

The electrodes are made from stainless steel pins, shaft diameter 0·15 mm (Watkins and Doncaster). A length of nickel-chromium wire, diameter approx. 0·03 mm, insulated with 'Diamel' (Johnson Mathey Metals Ltd) is attached at one end to the shaft of a pin and at the other to a male miniature connector (Amphenol) with electrically conducting adhesive (Eccobound Solder 72C; Emmerson and Cuming Inc.). The male connector is then plugged into a female connector on the gate of one of a pair of matched FETs (E415; Siliconix Ltd). The pins are glued together in a pair with Araldite (CIBA) to give a tip separation of 0·5 mm and then insulated except at the tip with three coats of Epoxylite 600 1-M (Epoxylite Corpn). INSL-X E-33 (INSL-X Products Corpn) is used to glue together the two wires from a pair of electrodes and to insulate the connectors on the transistors. Enamel insulated copper wire (diameter 0·05 mm; Bromley-Langton Ltd, Slough) can be used instead of Nickel-Chromium wire.

Each pair of FETs is part of a matched pair of source followers (Rosetto & Vandercar, 1972), the output of which is amplified differentially. Of the electrical components, only the FETs are carried by the animal, the remaining components being housed in a remote box and connected to the FETs with miniature multicore cable (Radiospares). Electrostatic interference is eliminated by embedding the transistor pair in 'Simplex'

denture repair material (Dental Fillings Ltd) and wrapping it in earthed aluminium foil.

The electrodes are implanted in the brain under Equithesin (Jen-Sal Inc.) anaesthesia (0·1 ml./50 g, I.P.) by a technique similar to that of Spooner (1965). The skull is exposed and a small area of it eroded using a dental drill. The electrodes are pushed through the skull at this point and fixed to the skull with De Trey's Zinc Cement (Amalgamated Dental Trade Distributors Ltd). An earth wire is attached to the comb and the transistors are sutured on to the skin of the back.

The following experiment was performed. Two pairs of electrodes were implanted in the right cerebral hemisphere, giving two recording channels, and one pair of electrodes was used to coagulate brain tissue around the electrode tips using a radiofrequency Lesion Maker (Grass LM3). After coagulation the potentials recorded from this pair of electrodes were greatly reduced. The chick was then allowed to recover from the anaesthetic. The e.e.g. recorded from the lesioned area was of very low amplitude. Neither from this nor the other recording electrode pair were potentials observed which coincided with body movements during running and vocalization. The chick was later anaesthetized for a second time and the lesioning electrodes transferred to the opposite hemisphere, where they recorded wave forms which appeared normal, suggesting that the lesioning process had not adversely affected the recording capabilities of the electrodes. Eye movements are unlikely to contribute to the e.e.g. wave forms, since removal of both eyes had no effect that could be detected by subjective assessment of the records from the anaesthetized chick.

The only artifact which has appeared on our records was due to vigorous shaking of the head, which occurs in some chicks. This source of artifact gives a characteristic wave form and the behaviour is clearly noticeable when the chick is continuously observed. The epochs of record containing this artifact can therefore easily be identified.

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The extent of the odontoblast process and dimensions of dentinal tubules in the cat

By G. R. Holland and I. P. Rogers. *Departments of Anatomy and Physiology (Oral Biology), University of Bristol, BS8 1DT*

Most of the recent electrophysiological investigations into the sensitivity of dentine have been carried out on cats but little information is available on the detailed histology of cat dentine and such as there is (Frank, Sauvage & Frank, 1972; Arwill, Edwall, Lilja, Olgart & Svensson, 1973) is not quantitative. This tissue presents a special problem in fixation and observations on the structure have been combined with efforts to determine whether conventional procedures can produce satisfactory preservation. Young adult cats anaesthetized with pentobarbitone were perfused with buffered aldehyde mixtures either for short periods of 60 min or less or for 4 hr. The canine teeth were removed and cut into a series of transverse blocks which were processed and flat embedded. Ultrathin sections were cut at different levels from the outer surface of the dentine towards the pulp. The sections were examined and photographed under the electron microscope. Measurements were made to determine the peripheral extent of the odontoblast process, the diameters of the tubules, the number of tubules per unit area and the proportion of the cross-sectional area occupied by the tubules. The odontoblast process seems to be limited to inner dentine after fixation of short duration and its apparent extent is not increased by prolonging the fixation. The dimensions of feline dentine are much smaller than those of human dentine.

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The use of the cobalt sulphide precipitation technique to delineate neuronal projections in the rat brain

By D. W. Lincoln and C. A. Mason. *Department of Anatomy, University of Bristol, BS8 1TD*

The techniques of cobalt sulphide precipitation (Pitman, Tweedle & Cohen, 1972) and axonal iontophoresis (Iles & Mulloney, 1971) have been modified for use on the vertebrate brain, employing the rat visual system as an experimental model. After cobaltous ions have been passed into neurones by iontophoresis through the axons of a transected nerve, the tissues are immersed in a dilute solution of ammonium sulphide to precipi-